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Immobilization of a Thermostable Inorganic Pyrophosphatase from the Archaeon *Pyrococcus furiosus* onto Amino-Functionalized Silica Beads

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ABSTRACT: This study focuses on the preparation and application of a recombinant thermophilic inorganic pyrophosphatase from the archaeon *Pyrococcus furiosus* on amino-functionalized silica beads. The amino-functionalized silica beads were prepared by coating with 3-aminopropyltriethoxysilane by silanization. The thermostable inorganic pyrophosphatase was rapidly and successfully immobilized onto the amino-functionalized silica beads with glutaraldehyde as a coupling agent (within 12 min, >95.4% protein was immobilized onto the support). The results show that the protein could be immobilized efficiently, with up to 1 mg of protein/g of support with 92.9% activity. Compared with the free enzyme, the immobilized enzyme displayed a high activity toward inorganic pyrophosphate, less sensitivity toward the pH, and increased thermal stability. The immobilized enzyme retained 56.9% of its initial activity after hydrolysis of the inorganic pyrophosphate after 12 consecutive cycles (total = 330 min) at high temperature; this indicated a high protein stability suitable for practical applications. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 40700.

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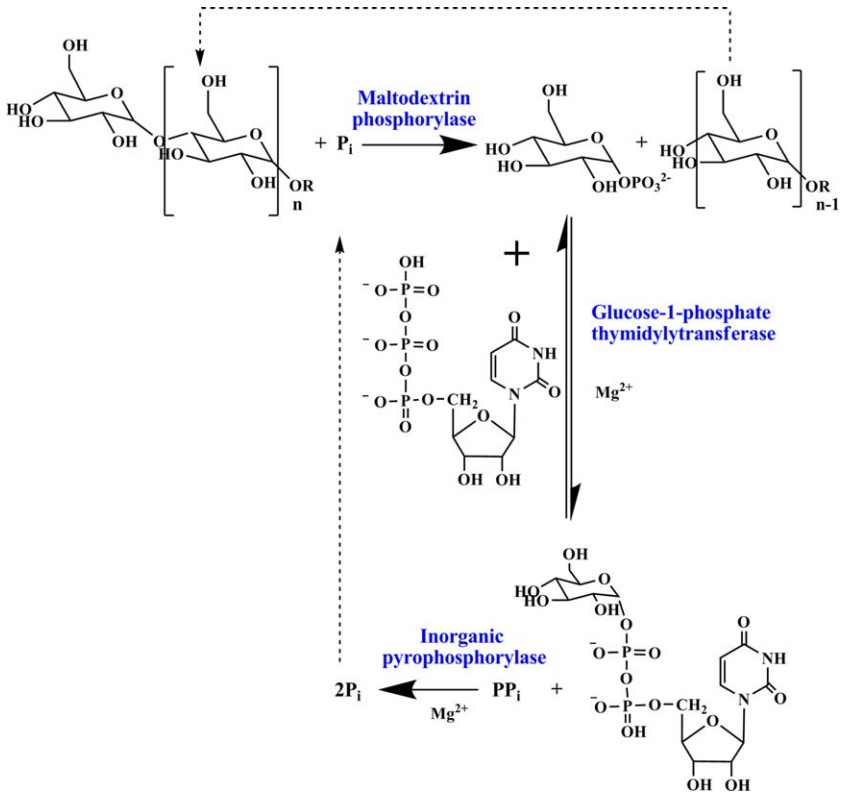
INTRODUCTION

The industrial enzyme business is steadily growing because of the many advantages presented by enzymes; these advantages include a high activity with high turnover frequencies under mild conditions, improved production technologies, tailorable enzyme properties, and new application fields.[1,2] However, the overall impact of enzymes on the industry remains considerably limited because of the high costs associated with their production, purification, and storage. Furthermore, most enzymes are deactivated under conditions considered to be beyond normal physiological ranges. Variations in the temperature, salt, pH, and solvent environments can significantly affect the enzyme activity.[3,4] For example, the optimum temperatures of most enzymes range from 20 to 50°C (37°C for mammalian enzymes), and most of these enzymes are heat-sensitive and easily deactivated. As thermophilic enzymes exhibit long half-lives and their optimum temperatures range from 50 to 120°C, interest in these enzymes has intensified, primarily because most existing industrial enzyme processes are conducted at high temperatures. A 10°C increase in the process temperature, for instance, can increase the reaction rate by twofold.[5,6]

The vast majority of enzymes are soluble in water. As such, the separation of the enzyme from the substrate or product is difficult in aqueous-phase reaction systems. In this case, enzyme immobilization may yield improved enzyme stability and high enzyme retention and recovery for the separation of the biocatalyst from the product. It can also prevent the carry-through of proteins or enzyme activity to subsequent process steps. Enzyme immobilization systems are conducive to continuous process development and beneficial and economical for biocatalytic processes. Therefore, compared with soluble enzymes, immobilized enzymes are the preferred biocatalyst for manufacturing-scale process operations.[7–9]

Silica beads are low-cost and heat-stable inorganic materials with excellent thermal stability, well-ordered porous structures, extremely high surface areas, and an abundance of hydroxyl groups. These beads have been reported to be excellent solid candidates for enzyme immobilization and biocatalytic applications.[10–12] In this study, raw silica beads were activated by reflux with methane sulfonic acid and were subsequently coated with 3-aminopropyltriethoxysilane (APTES) to achieve amino-functionalized silica beads. With the use of glutaraldehyde as the coupling agent, the aldehyde group of the protein was covalently attached to the surface of the amino-functionalized silica for immobilization. We believe that silica beads can provide excellent support for thermophilic enzyme immobilization.

Inorganic pyrophosphatase (enzyme classification (E.C.) 3.6.1.1), a ubiquitous enzyme, specifically catalyzes the hydrolysis of



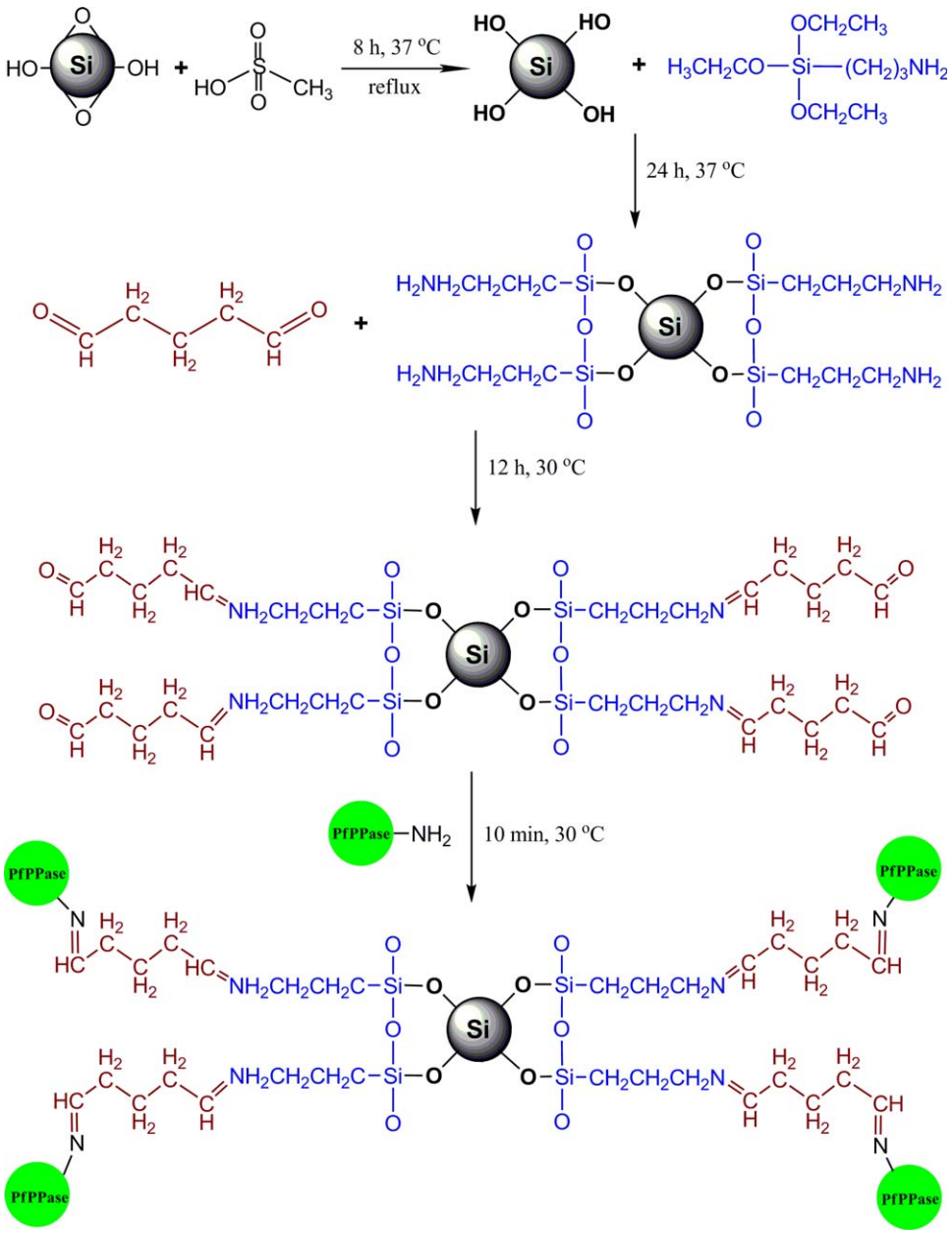
Scheme 1. Biosynthesis of uridine diphosphate glucose (UDP–Glc) through a one-pot reaction with multiple enzymes. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

pyrophosphate (PP_i) to orthophosphate (P_i), disrupts the enzymatic reaction equilibrium in the nucleotide metabolic pathway, and enriches useful products in the substrate. We previously reported the biocatalytic synthesis of diosgenyl-β-D-glucopyranoside and uridine diphosphate glucose via a method in which inorganic pyrophosphatase serves both to drive the reaction scheme in the uridine diphosphate glucose (UDP–glucose) direction and also to regenerate one starting product for the maltodextrin phosphorylase reaction[9,13] (Scheme 1). In addition, we chartered an extremely thermostable inorganic pyrophosphatase gene from the genome of hyperthermophilic archaeon *Pyrococcus furiosus* (PfPPase);[14] it has an optimum growth temperature of 100°C.[15] However, the immobilization of thermophilic enzymes for nucleotide metabolic pathways has rarely been reported. When PfPPase was immobilized onto amino-functionalized silica beads for consecutive cycle operation at high temperature (Scheme 2), it sharply cut down the production cost of PfPPase. In this study, the biochemical properties of immobilized PfPPase, the factors that influence its activity, and its application in the batch hydrolysis of inorganic PP_i were investigated in detail.

EXPERIMENTAL

Cloning, Expression, Purification, and Preparation of the Enzyme

The PfPPase gene (AE009950.1: AAL80381.1) was amplified from the template of *P. furiosus* (Deutsche Sammlung von Mikroorganismen 3638) genomic DNA in a 100-μL polymerase chain reaction (PCR) with forward 5′-ctggatccatgtcacaacctatttttaacg-3′ and reverse 5′-cttgcggccgcttagcgttttgcctgccag-3′ primers contained *Hind* III and *Not* I restriction sites, respectively. The DNA fragments (537 base pairs) amplified by the polymerase chain reaction were ligated into the expression vector **pET24a_(+).** The prepared *Escherichia coli* BL21 (DE3) cells containing pET24a–PfPPase were cultivated in a 250-mL flask containing 50 mL of Luria–Bertani medium containing 1% w/v bactotryptone, 0.5% w/v yeast extract, 1% w/v NaCl, and 50 μg/mL kanamycin at 37°C with shaking at 200 rpm. When the optical density at 600 nm (OD_{600}) of the culture broth reached about 0.7, 0.05 m*M* isopropyl-β-D-thiogalactoside was added, and the cultivation temperature was reduced and kept at 15°C with shaking at 120 rpm for 4 h to induce the expression of the target enzyme protein. The cells were harvested by centrifugation at 5000*g* for 10 min at 4°C and resuspended in lysis buffer [50 m*M* trishydroxymethylaminomethane (Tris)-HCl buffer, pH 7.0, containing 300 m*M* NaCl] followed by disruption by ultrasonication (400 W). The cell disruption fluid was immersed in an 85°C water bath for 5 min, cooled on ice for 10 min, and then centrifuged to eliminate denatured denatured proteins. The clarified supernatant was collected and further purified by an Ni-NTA His-Bind Purification Kit. A volume of 5 mL of the prepared and clarified supernatant was added to a purification column and bound for 30–60 min with gentle agitation to keep the resin suspended in the supernatant solution. The resin was settled by gravity and then washed with 5 mL of native wash buffer (per 50 mL of the native purification buffer with 20 m*M* imidazole, pH 7.0) three times. The native purification buffer contained 50 m*M* pH 7.0 Tris-HCl and 500



Scheme 2. Strategies for PfPPase immobilization onto amino-functionalized silica beads. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

m*M* NaCl. The proteins were eluted with 5–8 mL of native elution buffer (per 15 mL of native purification buffer with 250 m*M* imidazole at pH 7.0). After purification, the enzyme solution was demetalated by 50 m*M* ethylene diamine tetraacetic acid and dialysis. Then, the supernatant was collected and lyophilized to form a purified enzyme powder.

Protein and Enzyme Assays

Serial concentrations (0, 1, 0.5, 0.25, and 0.125 mg/mL) of standard protein solutions were quantified by the bicinchoninic acid kits as a calibrated curve. The absorbance at 562 nm was recorded. The concentration of the protein was calculated by the calibrated curve.

The assay mixture contained 50 m*M* Tris-HCl (pH 7.5), 5 m*M* $MgCl_2$, 10 m*M* $Na_4P_2O_7$, and 0.1 mg of PfPPase powder in a total volume of 1 mL.

The reaction mixture lacking the enzyme was used as a control. The reaction mixture was incubated at certain temperature for 1 min. After the reaction, 50 μL of the reaction mixture was withdrawn and transferred to the system that contained 10 μL of fresh phosphorus-determining reagent solution [17% H_2SO_4/2.5% Na_2MoO_4–$(NH_4)_2SO_4$/10% L-ascorbic acid/H_2O = 1:1:1:2] and 20 μL of H_2O. Then, the reaction was incubated at 45°C for 10 min. The absorbance at 660 nm was recorded. One unit of inorganic pyrophosphatase was defined as the activity to

catalyze 1 μmol of PP_i (2 μmol of phosphate) in 1 min under the conditions described previously. Specific activity was defined as units per milligram of protein. Each value was the mean of at least three independent determinations.

Preparation of the Amino-Functionalized Silica Beads

An amount of 20 g of naked 200 porous silica beads were acidized by the addition of 100 mL of 5% methanesulfonic acid in a 250-mL round flask by electromagnetic stirring for 8 h at 37°C. Then, acidated silica beads were washed by deionized water several times until a neutral pH was reached and harvested after vacuum drying at 30°C. After that, 20 g of acidated silica beads and 9 g of APTES were dissolved in 150 mL of ethanol/water (volume ratio = 1:1) solution. The solution was vigorously stirred at 37°C for 24 h. The synthesized amino-functionalized silica beads were then collected by centrifugation at 5000*g* for 5 min and washed with deionized water five times and then dried in a vacuum oven at 30°C. Certain amounts of amino-functionalized silica beads were suspended in Tris-HCl buffer (50 m*M*, pH 7.0). Thereafter, 25% glutaraldehyde (0.1 mL/mL) was added and shaken for 12 h at 30°C at 250 rpm. The amino-functionalized silica beads were then separated by centrifugation and washed five times with Tris-HCl buffer (50 m*M*, pH 7.0) to eliminate the excess unreacted glutaraldehyde.

Immobilization of PfPPase

Each enzyme solution was mixed separately with certain amounts of amino-functionalized silica beads and shaken at 30°C for 30 min at 250 rpm. Then, the immobilized enzyme was collected by centrifugation and washed five times with Tris-HCl buffer (50 m*M*, pH 7.0) to remove any unbound enzyme. The PfPPase binding efficiency (E) is defined as follows:

$$E=(C_1-C_0)/C_1 \quad (1)$$

where C_1 and C_0 are the amounts of PfPPase protein in the solution before and after immobilization, respectively. The activity recovery of the immobilized PfPPase (R) was calculated as follows:

$$R=A/(A_1-A_0) \quad (2)$$

where A is the activity of the immobilized PfPPase and A_1 and A_0 are the activities of the free PfPPase in solution before and after immobilization, respectively.

Comparison of the Free and Immobilized PfPPase

We optimized the activities of the free and immobilized PfPPase by running an enzymatic reaction according to the experimental designs at different temperatures and pH values. To investigate the effects of the pH on the free and immobilized PfPPase activity, the pH values (ranging from 7.0 to 13.0) were varied by 50 m*M* Tris-HCl buffer (from 7.0 to 9.0), 50 m*M* glycine (Gly)–NaOH buffer (from 8.5 to 10.5), 50 m*M* $NaHCO_3$–NaOH buffer (from 9.5 to 11.0), and 50 m*M* KCl–NaOH buffer (from 12.0 to 13.0) with 5 m*M* $Na_4P_2O_7$ and 5m*M* Mg^{2+} at 95°C, respectively. The thermal stabilities of the free and immobilized PfPPase were examined by the assay of their residual activities at 95°C after they were incubated in glycine (Gly)–NaOH buffer (50 m*M*, pH 10.0) at 85 and 100°C, respectively, for a required period. The apparent kinetic parameters, the Michaelis–Menten constant (K_m) and maximum reaction rate (V_{max}), of the free and immobilized PfPPase were determined by measurement of the initial reaction rate by the method described previously. K_m and V_{max} were calculated from Lineweaver–Burk plots according to the following formula:

$$1/V=K_m/V_{max}\cdot C_s+1/V_{max} \quad (3)$$

where V is the initial rate of reaction at different inorganic pyrophosphate concentrations (C_s's).

Repeated Hydrolysis of Inorganic PP_i with Immobilized PfPPase at a High Temperature

A 10-mL reaction mixture was performed with a substrate concentration of 5 m*M* with Tris-HCl buffer (50 m*M*, pH 10.0) at 95°C. The reactions were carried out at 95°C and 200 rpm in 25-mL equipped flasks. The product (phosphate) concentration was analyzed periodically by a phosphorus-determining reagent solution. When the conversion of substrate was nearly 100%, the reaction was terminated by centrifugation. The recovered immobilized enzyme was washed three times with Tris-HCl buffer (50 m*M*, pH 7.0) and was subsequently used again in the next batch of the reaction with fresh substrate.

RESULTS AND DISCUSSION

Characterization of Immobilized PfPPase

The recombinant soluble enzyme from the harvested cells was purified by heat treatment and Histidine (His)-trap affinity chromatography. The protein concentration was determined with a bicinchoninic acid (BCA) assay kit, and the purity was analyzed on a 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. A total of 80% of the expressed protein was soluble, and only a single band was observed after two-step purification.

The silica gel surface contained numerous silanol —OH and —O— strained siloxane groups. The subsequent groups could also be transformed into —OH by reflux with acid; this made the surface of the silica beads combine with more APTES molecules.[16] Studies have suggested methane sulfonic acid to be more efficient than hydrochloric acid.[17] Figure 1(A) shows the Fourier transform infrared (FTIR) spectra of both the raw and amino-functionalized silica beads. It is known that the stretching vibrations of $\nu_{C=O}$ in nonconjugated aldehyde appear at approximately 1700 cm^{-1}; the stretching vibrations of ν_{C-H} in aldehyde showed two peaks near at 2800 and 2700 cm^{-1}. The peaks at 2903.7 and 1665.9 cm^{-1} were only observed in Figure 1(B). The results provide convincing evidence that the aldehyde groups efficiently covered the surface of the silica beads.

Influence of PfPPase Immobilization

A concentration of 0.5 mg/mL free PfPPase solution was added to different concentrations of amino-functionalized silica beads. As shown in Figure 2 with an increase in the number of beads, the protein-binding efficiency was kept at approximately 100% until the concentration of the amino-functionalized silica beads was more than 0.5 g/mL. The binding efficiency of PfPPase was 95.4% with 1 mg/g carrier. Furthermore, the effect of the PfPPase coupling time on immobilization was investigated. Figure 2 shows that the process of PfPPase immobilization was instantaneous, and the protein was almost completely immobilized onto the aldehyde-functionalized silica beads in less than

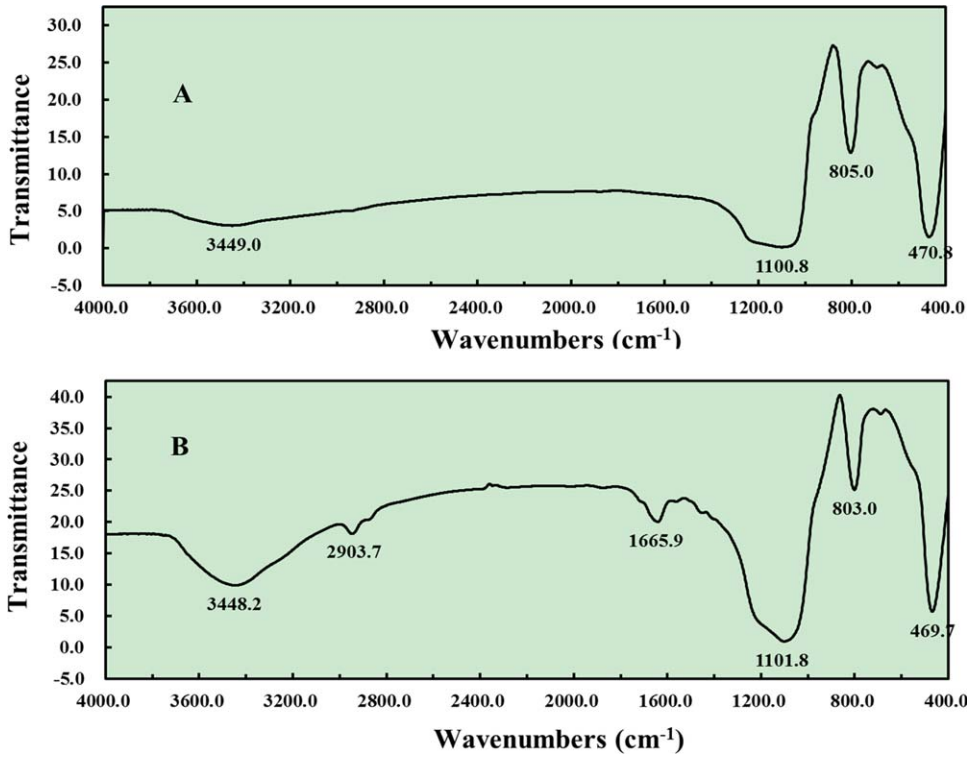


Figure 1. FTIR spectra of (A) raw and (B) amino-functionalized silica beads. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

12 min. It was reported that the enzyme immobilization may proceed first through rapid ionic exchange on the carrier, and covalent interactions follows the adsorption of the protein. The specific activity of the immobilized PfPPase was 51.7 U/g, and the recovered activity was 92.9%.

Properties of the Free and Immobilized PfPPase

Figure 3 shows a comparison between the free and immobilized PfPPase in response to the external pH. The overall trend of activity was similar for both the free and immobilized enzymes. However, the maximum activity of the immobilized PfPPase shifted slightly toward neutral pH. Furthermore, the immobilized PfPPase appeared to be more stable against pH changes than the free one, particularly from 7.0 to 9.0.

Figure 4 shows the comparison of the thermal stabilities of both the free and immobilized PfPPase. The half-life of the heat inactivation of free PfPPase was approximately 1901 and 489 min at 85 and 100°C, respectively. However, the half-life values of the immobilized enzyme was approximately 3268 and 992 min at 85 and 100°C, respectively. These results reveal that the thermal

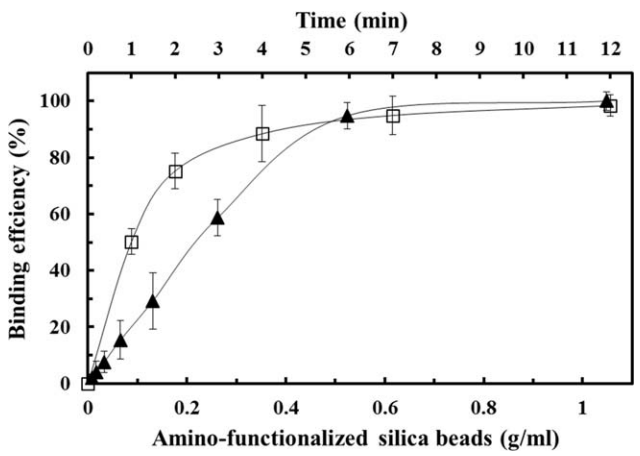


Figure 2. Effect of the amino-functionalized silica bead content on the PfPPase immobilization: (□) binding time and (▲) concentration of the amino-functionalized silica beads.

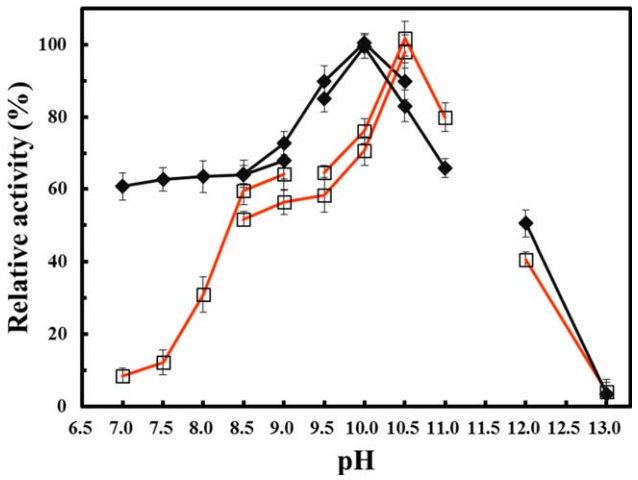


Figure 3. Effects of the pH on the initial activity of (□) free and (■) immobilized PfPPase. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

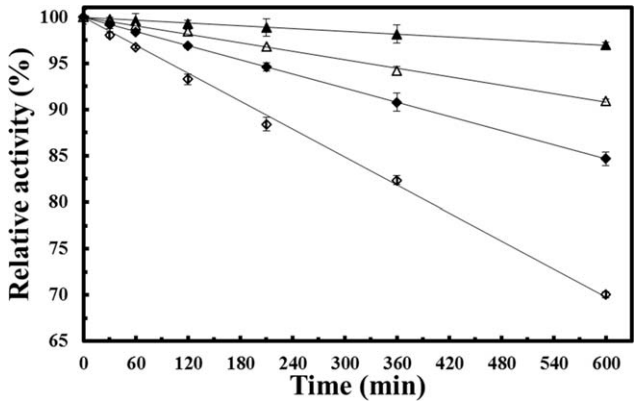


Figure 4. Thermal stability of the free PfPPase at (▲) 85 and (■) 100°C and immobilized PfPPase at (△) 85 and (□) 100°C.

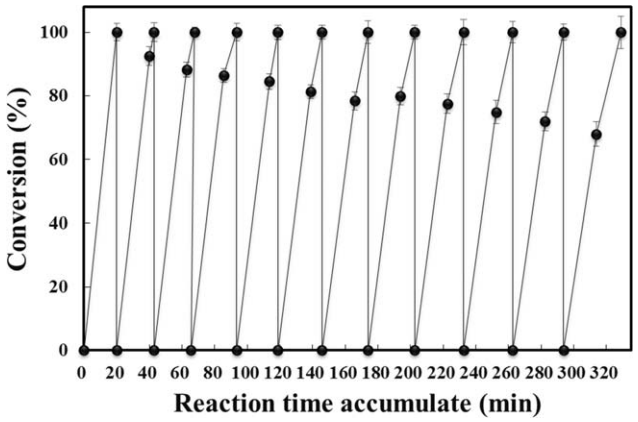


Figure 5. Repeated batch hydrolysis of inorganic PP_i with recycled immobilized PfPPase at 95°C.

stability of the immobilized PfPPase was significantly enhanced compared with the free enzyme. It was likely that the covalent immobilization made the PfPPase more rigid; thus, it became more resistant to heat inactivation.

The immobilization of the enzyme may result in variations in its kinetic behavior. The kinetic behavior of the free and immobilized PfPPase was examined at different inorganic PP_i concentrations, and the apparent K_m and V_{max} are presented in Table I. Because of some resistance from the carrier toward the interaction between the substrate and the enzyme, the K_m value of PfPPase increased almost 2.6 times compared with the free enzyme after immobilization, and the V_{max} value of the free PfPPase was almost 1.1 times higher than that of the immobilized one.

Reusability of the Immobilized PfPPase

The stability of the immobilized PfPPase during the hydrolysis of the inorganic PP_i was evaluated during 12 consecutive operation cycles (Figure 5). Despite the longer times of the following cycles, the immobilized PfPPase retained 56.9% of its initial activity after all of the batch reactions (ca. 330 min in total). Moreover, the protein and enzyme activities were barely assayed in the supernatant of each batch; this revealed that no protein was released into the reaction solution and confirmed the presence of strong covalent interactions. These results demonstrate that the immobilized PfPPase on the amino-functionalized silica beads possessed a high stability, even though the immobilizing process underwent an extremely fast rate and continuous operation at a high temperature.

CONCLUSIONS

In this study, low-cost amino-functionalized silica beads were synthesized with simple technology and used to immobilize the recombinant thermostable inorganic pyrophosphatase from the archaeon *P. furiosus*. The immobilization of PfPPase onto the aldehyde-functionalized silica beads was a rapid process, and the covalent immobilization with glutaraldehyde as the coupling agent elevated the protein-binding efficiency with a high activity. Compared with free enzyme, the immobilized PfPPase showed a higher thermal stability, less sensitivity to pH, a high stability, and reusability in 12 repeated batch reactions. Moreover, the aldehyde-functionalized silica beads showed excellent stability at high temperatures, no matter whether physical or chemical. Therefore, these results indicate that the aldehyde-functionalized silica beads could serve as an immobilization carrier support material for other thermophilic enzymes and suitable for practical applications. We will use amino-functionalized silica beads in a coming study to co-immobilize recombinant thermophilic maltodextrin phosphorylase, glucose-1-phosphate thymidylytransferase, and inorganic pyrophosphatase for a highly efficient and continuous synthesis of high-value additional uridine diphosphate glucose from maltodextrin and uridine triphosphate (UTP) in one pot at a high temperature.

Table I. Kinetic Parameters of the Free and Immobilized PfPPase for Inorganic PP_i

PfPPase	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})
Free	0.175	55.6
Immobilized	0.451	51.7

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